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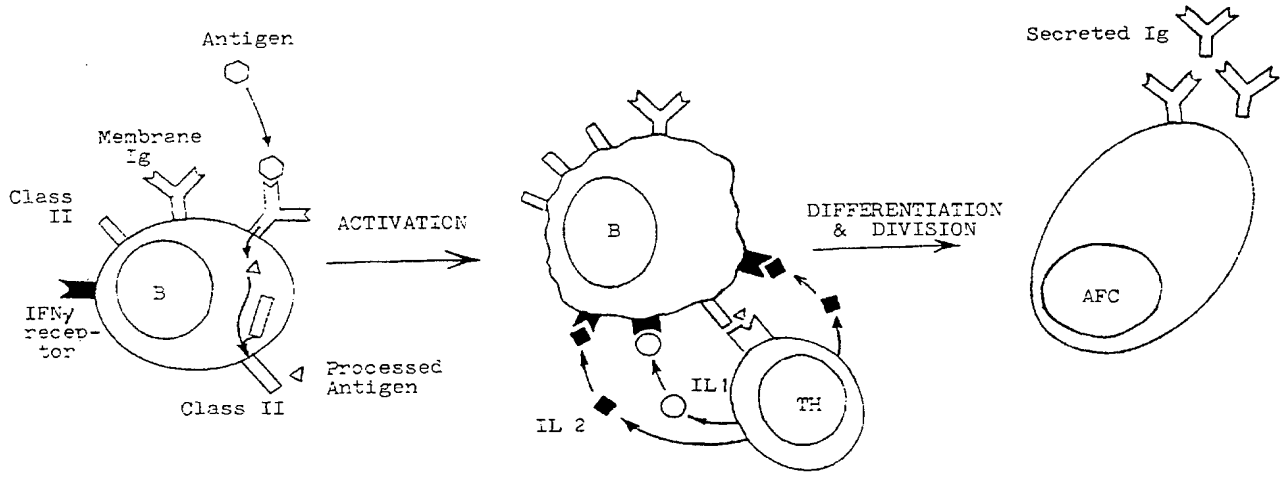

(57) Abstract

Antibodies to haptens and other B cell antigens are provided in animals by a process wherein the B cell antigen is introduced into the animal in a form in which it is coupled to an immunogenic carrier molecule in the form of a protein antigen which normally induces both T cell and B cell immunity. The process is unique in that the immunogenic protein antigen is modified to an exclusive or predominant T cell antigen by a denaturating modification of the antigen, that the B cell antigen to which antibodies are to be provided is coupled to the modified T cell antigen, followed by immunization, optionally using an adjuvant as an immune stimulating means, preferably after preimmunization with T cell antigen in a form that may be different from the form used in the immunization proper with the B cell antigen. The produced antigens are particularly used in the production of vaccines for veterinary medicinal use.

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Method of producing antibodies with conjugates of B-cell-antigen and denatured carrier.

The present invention concerns a process for providing antibodies to haptens and other B cell antigens as well as antibodies obtained by the process. The invention moreover concerns the use of the produced antibodies for vaccines, in particular for veterinary medicinal use.

The general immune response is based on a cooperation between antigen presenting cells, antigen specific T lymphocytes and antigen specific B lymphocytes. The T lymphocytes are selected and stimulated by T cell antigens which, associated with MHC class II (Major Histocompatibility Complex) molecules, are presented on the surface of so-called antigen presenting cells; see figure 1.

A T cell antigen (a T cell epitope) can be described by the following characteristics:

1) A short, linear amino acid sequence.

2) Most frequently localized in the interior portion of a protein and thus inaccessible on the surface.

3) Reacts with the antigen specific receptor of the T cell only when it is bound to an MHC class II molecule (thus forms part of a ternary complex).

The B lymphocytes are selected by the administered antigen which binds to the antigen specific receptor (membrane bound antibody) of the B cell. Stimulation of the selected B cell takes place by internalizing and processing the antigen, following which the T cell epitope of the antigen is presented on the surface of the B cell, associated with

MHC class II molecules. This presentation entails that the selected and stimulated T accessory cells are associated with the antigen specific B lymphocyte, which is now stimulated and matured via hormone-like factors from the T cell (e.g. interleukins). The final stage of the B cell after stimulation and maturing is the antibody producing plasma cell which secretes antigen specific antibody; see figure 2.

A B cell antigen (a B cell epitope) can be described by the following characteristics:

1) Substantially any molecular configuration can act as a B cell antigen (epitope). As regards proteins, these epitopes are most frequently positioned on the outer, accessible face of the protein.

2) A B cell antigen (epitope) can bind directly to the antigen specific receptor (the antibody) of the B cell.

Immune response in the form of antibody formation to a given antigen is therefore conditional upon the presence of both B cell epitope(s) and T cell epitope(s). These two types of epitopes must be physically associated. The basis of this knowledge are classic experiments from around 1970 concerning the hapten carrier molecule problems (see K. Rajewsky et al., J. Exp. Med. 129, 1131 (1969), N.A. Mitchison, Eur. J. Immunol. 1, 10 (1971) and N.A. Mitchison, ibid. 1, 18 (1971)).

Antibody response to low molecular antigen molecules (haptens) can be induced in a mammal only if the hapten presented for the animal in question is coupled on an immunogenic carrier. Another phenomenon which frequently constitutes a problem is that certain molecules, which otherwise

have a relatively large molecular size, are only very little immunogenic. This may apply to e.g. larger peptides, low immunogenic proteins, carbohydrates, lipids, nucleic acids, etc.

Examples of carrier molecules useful for inducing or enhancing the immune response to such molecules are serum albumin (BSA, HSA and others), keyhole limpet hemocyanin (KLH), ovalbumin (OA), chicken immunoglobulin (ChIg) and diphtheria toxoid (DT), but a large number of others might be mentioned.

It has now surprisingly been found that an immunogenic protein antigen, which induces both T cell and B cell immunity under normal circumstances, can be converted to or act as an immunogenic, "pure" T cell antigen to which no or only very little B cell immunity is induced. Then, the molecule to which antibodies are to be produced, can be coupled chemically to this T cell antigen or to a corresponding, modified T cell antigen.

This provides the following advantages:

a) T cell immunity can be induced as a general principle in an animal. This can be done while very limited B cell immunity or none at all is induced in relation to the carrier molecule which is subsequently used for induction of the relevant antibody response to hapten or the like.

b) Subsequent immunization with a B cell antigen (hapten or the like), coupled to a modified form of the above-mentioned T cell antigen, ensures:

1) rapid development of antibodies,
2) a high level of antibodies already after one or two immunizations,
3) sustained immune response, and
4) possibility of rapid development of antibodies having a high binding strength.

c) Immune response in the form of antibody formation to the T cell antigen (carrier molecule) can be "optimized" to be very low (possibly not measurable).

More particularly, the invention concerns a process for providing antibodies to haptens, peptides/proteins, carbohydrates, lipids, nucleic acids, weak immunogens and other B cell antigens, including combinations of these, in animals, wherein the B cell antigen introduced in the animal is coupled to an immunogenic carrier molecule in the form of a protein antigen, which normally induces T cell immunity as well as B cell immunity. The process of the invention is characterized by modifying the immunogenic protein antigen to an exclusive or predominant T cell antigen by a denaturating modification of the antigen, coupling the B cell antigen to which antibodies are to be produced to the modified T cell antigen, followed by immunization, optionally using an adjuvant as an immune stimulating means, preferably after preimmunization with T cell antigen in a form which may be different from the form used in the immunization proper with the B cell antigen.

The invention further concerns antibodies or antisera obtained by the present process. Finally, the invention concerns use of the antigens produced by the process for the production of vaccines, in particular for veterinary medicinal use.

Processes which are related to the process according to the invention for providing antibodies to B cell antigens in animals, are known from EP published specification No. 0429816 and from Science 249, 423-425 (1990). These literature sources concern a structure which represents a T accessory epitope and which contains information for a carrier function, but not for a B epitope suppressive function. These T cell structures may stem from the same protein, in which case it will be necessary to remove sequences which contain information for the suppressive function, or alternatively to change these sequences in such a way that they no longer exhibit suppressive function. The T cell epitope may be a sub-part of various structures, especially parts or microorganisms, in particular sequences from tetanus toxoid. It is stated nowhere that the T cell antigen may be denatured whole antigen.

WO published specification No. 89/06974 concerns T cell epitopes from bacterial products and describes how a T cell epitope is defined, isolated, produced and coupled to a B cell epitope. The specification which especially describes bacterial toxines, does not mention a general use of denatured proteins as carrier molecules.

Nor is this so in case of EP published specification No. 0378881 and No. 0427347 which both describe synthetic peptides and their use as universal carriers for use in the production of immunogenic conjugates suited for development of synthetic vaccines. A survey of the production of antigenic hapten-carrier conjugates can be found in the article by B. Erlanger: "The preparation of Antigenic Hapten-Carrier Conjugates: A Survey", Methods in Enzymology 70, 85-104 (1980). This article is, however, a description of the fundamental hapten-carrier problems and in no way refers to the complex of problems

concerning native/denatured proteins.

By the process according to the invention the modification of the immunogenic protein antigen to exclusive or predominant T-cell-antigen is performed, as opposed to the known technique, by denaturation, whereby the advantages stated above under (a)-(c) are obtained.

The process of the invention is based on the following elements:

A. Conversion of a combined T/B cell antigen to a predominant T cell antigen

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This procedure involves modification of a protein antigen, e.g. in the form of a so-called denaturation of the antigen. If the modification is effected as a denaturation, this may take place by common methods for achieving denaturation, but preferably by methods comprising blocking charged groups (either $-NH_3^+$ or $-COO^-$), e.g. for $-NH_3^+$ by means of formaldehyde, and reducing disulfide bridges, e.g. by means of dithiothreitol or β-mercapto ethanol. Then the actual denaturation takes place by e.g. heat treatment. The T cell antigen then occurs in two forms: a non-modified one and a modified one.

B. Coupling of the B cell antigen to the T cell antigen

This coupling takes place e.g. in one of the following manners:

a. The hapten is modified chemically, so that e.g. a carboxylic, a sulfone or other acid group is converted to an active derivative, e.g. an active ester, an acid halo-

genide or an acid anhydride, symmetrically or mixed, which then reacts with the carrier protein with covalent coupling. Other cases may involve activating a mercapto group by converting it to an activated disulfide, which is then caused to react with the mercapto group on the carrier protein (see the following).

b. In many cases the native hapten does not contain a group which can be converted to an activated derivative. This may be handled by modifying the hapten, e.g. by introducing a carboxyl group or mercapto group which can then be activated by introducing ligands with an α,β-activated double bond, such as e.g. vinyl sulfonyl, or by introducing a photochemically activatable group, such as e.g. 4-azidobenzoyl or 2- or 3-diazocyclopentadienyl carbonyl; these couplings may also be established via Hg-S bonds.

c. If the carrier protein is not equipped with the necessary functional groups, such as e.g. SH, these groups may be introduced chemically before the coupling step takes place.

d. Carrier proteins can be modified so that their functional groups are activated, e.g. by converting carboxylic acid groups to active esters or by activating mercapto groups so that these can be coupled to the functional groups of the hapten.

e. Hapten and carrier protein are caused to react with homo- or heterobifunctional groups, such as e.g. divinyl sulfone, dicarboxylic acids, formaldehyde, glutaraldehyde or glutaconaldehyde, active esters of dicarboxylic acids, maleimide active esters, 4-azidobenzoyl derivatives of active esters, etc.

C. Immunization

Immunization may comprise preimmunization with T cell antigen in a form different from the form which is used in the actual immunization with the B cell antigen. This is followed by the actual immunization with the B cell antigen coupled on a relevant T cell antigen. An adjuvant may be used for supporting the immunization.

The immunization path may be selected from several useful immunization paths.

Preimmunization takes place under circumstances where the humoral immune response to the subsequently employed carrier molecule (antibody formation) is as low as possible, while inducing T cell immunity to the subsequently employed carrier molecule.

The actual immunization takes place with the B cell antigen coupled on the carrier molecule (the T cell antigen in modified form compared with the preimmunization). The immunization typically comprises the use of an adjuvant as an immune stimulating means, such as e.g. aluminium hydroxide, Freund's adjuvant (complete or incomplete), saponin or another known adjuvant.

The type of B cell antigen may be any molecular configuration to which antibodies can be produced, e.g. a hapten, a peptide of any size, a carbohydrate, a lipid, a nucleic acid molecule, a protein having low immunogenecity or a combination of these.

The coupling chemistry should be well-defined as far as possible, i.e. the reactions taking place must be understandable in a molecular sense, and they must be control-

lable as follows:

a. B cell antigen is selectively coupled to the carrier protein, whereas formation of di- and polymeric aggregates is obviated, i.e. no or little formation of carrier-carrier or B cell antigen B cell antigen aggregates.

b. It should preferably be possible to control the coupling density so that the coupling is really irreversible under the experimental conditions.

The invention will be illustrated more fully by the following examples.

EXAMPLE 1

Carrier modifications

When subjected to denaturing impacts, proteins tend to form large aggregates, primarily because of the establishment of intermolecular disulfide bridges, but also because of the establishment of non-covalent interactions between molecules.

This tendency can be counteracted/eliminated by various chemical methods, such as e.g. reduction of disulfide bridges (e.g. with dithiotreitol) followed by alkylation (e.g. with iodine acetamide), or by modification of -COOH groups or $-NH_2$ groups. Then e.g. a denaturing heat treatment can be performed without causing precipitation.

The conversion of a native protein to a heat-denatured protein by means of formaldehyde treatment (which "blocks" amino groups) followed by denaturing heat treatment is described as an example.

The protein solution is adjusted to 3 mg/ml in an 0.1M phosphate buffer, pH 7.4. Lysine HCl is added to this protein solution, corresponding to 4.57 mg of lysine HCl per ml of protein solution. 37% formaldehyde (Merck 4002) is now added slowly with stirring, corresponding to 4 µl of formaldehyde per ml of protein solution.

After sterile filtration (0.22 µm) the mixture is incubated at 37°C with slow stirring for 4 weeks.

The solution is finally autoclaved for 1 hour at 105°C. The solution is centrifuged (10,000 g) for 20 minutes, and the supernatant from this is dialysed against the desired buffer, following which the protein solution is sterile filtrated (0.22 µm) and is stored at 4°C in a suitable volume.

The protein concentration is determined by the method described by Bradford (Bradford, M.M., Anal. Biochem. 22, 248, 1876).

EXAMPLE 2

The immunogenicity of native and denatured, respectively, antigen (carrier), evaluated by antibody formation to the antigen after immunization with various doses of antigen

Groups of mice (5 in each) were immunized with various doses of native antigen (ovalbumin), from 0.01 µg per immunization to 100 µg per immunization (fig. 3A), or with various doses of denatured antigen (ovalbumin) (fig. 3B). In each immunization the antigen was administered adsorbed to aluminium hydroxide, ($Al(OH)_3$). The immunizations were repeated at intervals of 14 days, and blood samples were taken 10 days after each immunization for measurement of antibody amounts (titer).

The antibody amount (the titer) was measured by an ELISA method in which the antigen was applied to polystyrene plates. Sera were diluted 1:1000, from which twofold dilutions were performed. The titer can be evaluated on the basis of the serum dilution where the signal value (the ordinate) is 50% of the maximum signal value.

As an example, the figure shows the antibody response in the various groups after the 5th immunization. It will be seen that the threshold value for a positive response after immunization with native antigen is 0.01 µg/immunization, whereas it is 0.5-1 µg/immunization after immunization with denatured antigen.

Thus, the immunogenicity of native antigen is 50-100 times higher than that of denatured antigen.

EXAMPLE 3

Antibody response to hapten and to carrier, respectively, at a given hapten density, but using various doses of antigen for immunization (evaluated as the amount of carrier protein)

An N-hydroxysuccinimide activated hapten (9-thioguanine acetic acid) was coupled to heat denatured ovalbumin (OA) in a carbonate buffer, pH 8.0. 10 µl of a solution of NHS-9-thioguanine acetic acid in DMSO, 40 mg/ml, were added with slow stirring to a solution of OA whose concentration was 1 mg/ml.

Groups of 5 mice each were then immunized with various doses of antigen, adsorped to $Al(OH)_3$ as adjuvant. A total amount of 200 µl of vaccine, of which 2 mg/ml were adjuvant, was given in each immunization, which was given intraperitoneally).

The mice were bled 10 days after each immunization (which took place at intervals of 14 days), and mouse sera were analyzed for antibody titer by an ELISA technique, partly to the hapten thioguanine, partly to the carrier, OA.

Figure 4 shows that there is a poor or low antibody titer to the carrier molecule at low doses of the antigen, and that at selected antigen doses it is only possible to obtain antibodies to the hapten.

EXAMPLE 4

Relation between antibody response to the hapten and antibody response to the carrier molecule, partly using a native protein as a carrier molecule, partly using a denatured protein as a carrier molecule

The A-part of figure 5 shows the antibody response in mice, immunized with a hapten (merthiolate) coupled to native ovalbumin as a carrier molecule. ---X--- shows the antibody response to the hapten, while ---o--- shows the antibody response to the carrier part. 0.5 µg of antigen per immunization is used in the immunization. The figure shows the antibody response after 3 immunizations.

The B-part of figure 5 shows the antibody response after immunization with hapten (merthiolate) coupled on denatured antigen (ovalbumin). ---x--- shows the antibody response to the hapten, while ---o--- shows the antibody response to the carrier part. 0.5 µg of antigen per immunization is used in the immunization. The figure shows the antibody response after the 3rd immunization.

It appears from figure 5 that an immunization dose with denatured carrier protein where no antibody response is developed to the carrier part, but only to the hapten, can

be selected from a dose response graph (see examples 2 and 3).

EXAMPLE 5

The importance of the coupling degree of a hapten on a carrier molecule, partly for the antibody response to the hapten, partly for the antibody response to the carrier part

Groups of mice with 5 mice in each group were immunized with a carrier molecule (denatured ovalbumin, 10 µg per immunization), to which various amounts of activated hapten (merthiolate) were coupled (indicated in figure 6 as the amount (µl) of N-hydroxysuccinimide ester of merthiolate (40 mg/ml) in DMSO, added to 1 ml of denatured ovalbumin, 1 mg per ml in carbonate buffer, pH 8.0). After coupling the individual antigens were dialysed against PBS, and then they were adsorbed on adjuvant ($Al(OH)_3$) and used for the immunizations.

As appears from figure 6, the test comprised the use of from 0 to 100 µl of active merthiolate in the couplings.

The antibody response to hapten was analysed by an ELISA technique in which hapten (merthiolate) was used for the titration, coupled to an irrelevant carrier (diphtheria toxoid) for measurement of the hapten specific response, while uncoupled ovalbumin was used for the measurement of the antibody response to carrier (ovalbumin).

In figure 6 the antibody response is indicated in arbitrary units, the antibody response to uncoupled carrier being put at 100 for the carrier reponse, while for the hapten response the maximum response after immunization with carrier, coupled with 100 µl of active methiolate, is

put at 100.

It appears from figure 6 that an increase in the carrier density (coupling) results in an increased response to the hapten to a certain limit, while the antibody response to the carrier part constantly diminishes.

Thus, the selection of an immunization dose of carrier providing a significant antibody to uncoupled carrier and the increase in the hapten density result in a higher antibody response to hapten, while the anti carrier antibody response will be low or non-existing.

(NB: here it is also possible to use native carrier and to obtain the same effect in principle, but the increased coupling will give an ever greater denaturation).

EXAMPLE 6

Photoconjugation, including immunization and effect on antibody response

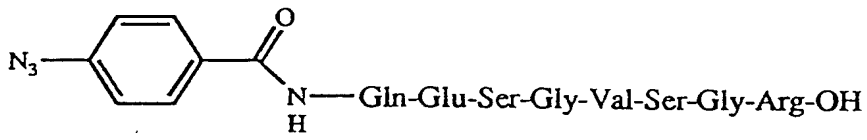
a) Synthesis of the octamer peptide KU834

H-Gln-Glu-Ser-Gly-Val-Ser-Gly-Arg-OH

The peptide was synthetized manually on 1.5 g of an Fmoc-Arg(Mtr)-PepSyn KA resin using standard Fmoc strategy with either Pfp ester or BOP couplings. The side chains were protected with t-butyl based groups with the exception of arginine which was protected with Mtr. The peptide was separated from the solid carrier by treatment of 742 mg of the peptidyl resin with 93:2:2:2:1 TFA/H_2O/thioanisole/thiophenol/ethanedithiol for 16 hours. After filtration the peptide was isolated by reducing the volume from the TFA mixture in a nitrogen current and then precipitated

with diethyl ether. The precipitated peptide was redissolved in 0.1% TFA and freeze dried, following which it was dissolved again in 0.1% TFA and purified by reverse phase chromatography. The purified peptide eluted as a single peak by reverse phase HPLC and was further identified by mass spectrometry: (M+H) 819.8.

b) Synthesis of the octamer 4-azidobenzoyl peptide KU843



750 mg of the peptidyl resin were treated with 3 equivalents of p-azidobenzoic acid ester dissolved in DMF to ninhydrin negative reaction. After washing and drying of the resin the crude 4-azidobenzoyl peptide was recovered and purified as described for KU834 above. The purified 4-azidobenzoyl peptide eluted as a single peak by reverse phase HPLC and was further identified by mass spectrometry (M+H).

c) Coupling of carrier protein

KU834 was coupled to the carrier protein in five different peptide carrier ratios (mole:mole):

1) peptide:carrier 1:1
2) peptide:carrier 4:1
3) peptide:carrier 5:1
4) peptide:carrier 10:1
5) peptide:carrier 50:1

Peptide and carrier in 0.1 M phosphate buffered saline (PBS), pH 7.5, were mixed in the desired coupling ratio. A corresponding volume 0.2% glutaraldehyde in 0.1 M PBS, pH 7.5, was added over 15 minutes. The coupling mixture was left to stand for 48 hours (end-over-end) at 4°C.

KU843 was photocoupled to the carrier protein in five different peptide carrier ratios (mole:mole):

1) peptide:carrier 1:1
2) peptide:carrier 4:1
3) peptide:carrier 5:1
4) peptide:carrier 10:1
5) peptide:carrier 50:1

Peptide and carrier in 0.1 M $NaHCO_3$ were mixed in the desired coupling ratio, and the mixture was then illuminated for 30 minuttes in a photochemical Rayonet reactor (16 UV lamps, wavelength 350 nm).

EXAMPLE 7

Thioguamine active ester

a) 2-Amino-9-carboxymethyl-6-chloropurine and 2-amino-7-carboxymethyl-6-chloropurine

Bromine acetic acid (3.0 g; 21.7 mmoles) and anhydrous K_2CO_3 (8.3 g; 60 mmoles) were mixed in anhydrous DMF (30 ml) with stirring, and 2-amino-6-chloropurine (3.1 g; 18.1 mmoles) was added. Water (about 100 ml) was added after 3 hours at room temperature, and the 9-isomer (2.4 g; 10.55 mmoles; 58%) precipitated by addition of concentrated HCl until pH 3. Further addition of water followed by cooling caused the 7-isomer to precipitate (0.5 g; 2.2 mmoles; 12%, contaminated with a little 9-isomer). The resulting

9- and 7-isomers can be separated on a silica gel column. Elution wity EtOAc:MeOH (0-50%) first gave the 9-isomer and then the 7-isomer. MS (Fab+) m/e 228 (M+1). Analysis calculated for $C_7H_6ClN_5O_2.0.5\ H_2O$: C, 35.53; H 2.98; N, 29.59; Cl 14.98. Found C, 35.51; H 2.69; N 28.90; Cl, 15.07.

b) 2-Amino-9-carboxymethyl-6-purinethiol

2-Amino-9-carboxymethyl-6-chloropurine (410 mg; 1.8 mmoles) and thiourea (137 mg; 1.8 mmoles) were mixed in EtOH (20 ml) and refluxed for 1 hour with stirring. The product (350 mg; 1.56 mmoles, 86%) was isolated after cooling and then suspended in water (25 ml). Solid $NaHCO_3$ was added, and then the solution was filtered and admixed with concentrated HCl until pH 3, whereby the analysed product precipitated. MS (Fab+) m/e 226 (M+1). Analysis calculated for $C_7H_7N_5O_2S.0.5\ H_2O$: C, 35.89; H 3.44; N, 30.03; S, 13.69. Found: C, 35.29; H, 3.15; N, 29.92; S, 13.82.

c) Succinimidylthioguanine-9-yl acetate

2-Amino-9-carboxymethyl-6-purinethiol (225 mg, 1 mmole) and N-hydroxysuccinimide (NHS) (138 mg; 1.2 mmoles) was dissolved in anhydrous DMF (5 ml). N,N'-dicyclohexylcarbodiimide (DCC) (247 mg; 1.2 mmoles) was added, and then the reaction mixture was left to stand overnight at room temperature. The resulting N,N'-dicyclohexylurea (DCU) was filtered off, and the product was precipitated with ether, collected and washed with methanol and ether (155 mg; 0.48 mmoles, 48%). The product hydrolyzes easily and could therefore not be purified additionally. MS (Fab+) m/e 323 (M+1).

EXAMPLE 8

The importance of preimmunization with carrier for achievement of antibody response to hapten

Groups of 5 mice were preimmunized with native carrier, or they received no preimmunization, and then they were immunized 14 days later with hapten coupled on native and denatured carrier, respectively. 25 µg of carrier were used for all immunizations; see figure 7.

The example shows that when the same carrier conformation (here native) is used for preimmunization as well as for immunization, the antibody response to hapten (merthiolate) is suppressed compared with the situation where no preimmunization has taken place. If, on the other hand, a different carrier conformation is used for preimmunization and for the immunization with the hapten coupled composition, the antibody response to hapten is increased compared with the situation where preimmunization has not taken place.

EXAMPLE 9

Importance of preimmunization of animals with carrier protein. Evaluation of the optimal time that may elapse from preimmunization with carrier until immunization with hapten carrier conjugate

Groups of 5 mice each were preimmunized with 25 µg of native carrier followed by varying time intervals (1 week to 12 months) of immunization with hapten coupled denatured carrier. Merthiolate was used as hapten and OA as carrier in the test. Immunization dose was 25 µg of hapten carrier

complex.

Blood samples were taken 14 days after the 1st immunization with the hapten carrier complex, and these were analyzed for antihapten antibody content.

The antihapten titer in the group, which had been preimmunized with native antigen 1 month before immunization with hapten carrier complex, has been given the value 100 arbitrary units.

Animals which had not been preimmunized had an antibody titer of 5 AU.

It will be seen from figure 8 that the period 1-4 months after preimmunization with carrier is optimum, but there is a distinct effect even after 12 months.

EXAMPLE 10

T-cell-presentation of native and denatured antigen, respectively, by means of antigen-incubated (pulsed) macrophages

Peritoneal macrophages were isolated from 20 CF1xBalb/c-mice. The macrophages were washed in a serum-free medium (DMEM), adjusted to a cell density of $2x10^6$ ml and incubated (pulsed) for 20 minutes at 37°C with antigen. Three groups (A, B and C) were incubated with native ovalbumin (OA), 2 mg/ml, denatured OA, 2 mg/ml, and DMEM without antigen, respectively.

After incubation the cells were washed 3 times in cold (4°C) medium, and finally they were resuspended in DMEM to a final cell density of 10^7/ml.

From each of the groups A, B and C, 1 ml (10^7) cells were injected intraperitoneally, so that mouse group I received cells pulsed with native OA, mouse group II received cells pulsed with denatured OA, while mouse group III received cells which had only been incubated with medium without antigen.

After 14 days 3 mice from each mouse group were immunized intraperitoneally with 100 µg native OA together with $AI(OH)_3$, a total of 0.5 ml, while the remaining 3 were immunized intraperitoneally with 100 µm denatured OA with $AI(OH)_3$.

Three weeks later blood samples were taken from all the mice, and sera were analyzed for antibodies to denatured OA in the mice which had been ip-immunized with denatured OA, and to native OA in the mice which had been ip-immunized with native OA, respectively.

Macrophages, pulsed with:	Antibodies to denatured OA	Antibodies to native OA
Native OA	++	+++
Denatured OA	++	++
Medium without antigen	-	-

The experiment shows that mice which have been given macrophages, either pulsed with native or with denatured OA, will both develop T cell immunity capable of supporting immune response to both native and

denatured OA.

This example confirms the assumption that native and denatured antigen both contain T cell epitopes capable of stimulating T accessory cells, and that these then subsequently can assist an antibody response to either the native or the denatured antigen.

Patent Claims:

1. A process for providing antibodies to haptens, peptides proteins, carbohydrates, lipids, nucleic acids, weak immunogens and other B cell antigens, including combinations of these, in animals, wherein the B cell antigen is introduced into the animal coupled to an immunogenic carrier molecule in the form of a protein antigen which will normally induce both T cell immunity and B cell immunity, characterized by modifying the immunogenic protein antigen to an exclusive or predominant T cell antigen by a denaturating modification of the antigen, coupling the B cell antigen to which antibodies are to be provided to the modified T cell antigen, followed by immunization, optionally using an adjuvant as an immune stimulating means, preferably after preimmunization with T cell antigen in a form which may be different from the form used in the immunization proper with the B cell antigen.

2. A process according to claim 1, characterized in that the actual denaturation is preceded by blocking of charged groups, e.g. by reaction with formaldehyde or gluteraldehyde in case of $-NH_3^+$, or with carbodiimide in case of $-COO^-$, and/or reduction of S-S groups followed by alkylation of free HS groups, and then the denaturation proper takes place in a manner known per se, e.g. by heat treatment.

3. A process according to claim 1, characterized in that coupling of the B cell antigen to the T cell antigen takes place by chemical modification of the B cell antigen to form an active derivative, which then reacts with the protein antigen serving as an immunogenic carrier molecule, with formation of a covalent bond or by

chemical modification of the T cell antigen to form an active derivative which then reacts with the B cell antigen with formation of a covalent bond.

4. A process according to claim 3, c h a r a c t e r i z e d in that the B cell antigen or the T cell antigen is modified, e.g. by introducing carboxyl or mercapto groups, which can then be activated, e.g. by introduction of ligands having an α,β-activated double bond or by introduction of a photochemically activatable group, such as 4-acidobenzoyl or 2- or 3-diazocyclopentadienyl carbonyl.

5. A process according to claim 1, c h a r a c t e r i z e d by using in the immunization an immunological adjuvant, e.g. aluminium hydroxide, complete or incomplete Freund's adjuvant or saponin.

6. A process according to any of claims 1-5, c h a r a c t e r i z e d in that the B cell antigen is selected from haptens, smaller peptides consisting of about 2-12 amino acids, larger peptides consisting of about 65 amino acids and proteins having low immunogenicity.

7. A process according to any of claims 1-5, c h a r a c t e r i z e d in that the B cell antigen is selected from peptides consisting of about 13-30 amino acids.

8. A process according to any of claims 1-5, c h a r a c t e r i z e d in that the B cell antigen is selected from peptides consisting of about 50 amino acids.

9. Antibodies or antisera, c h a r a c t e r i z e d in that they are produced by a process according to any of the preceding claims.

10. Use of antigens produced by a process according to any of claims 1-6 for the production of vaccines, in particular for veterinary medicinal use.

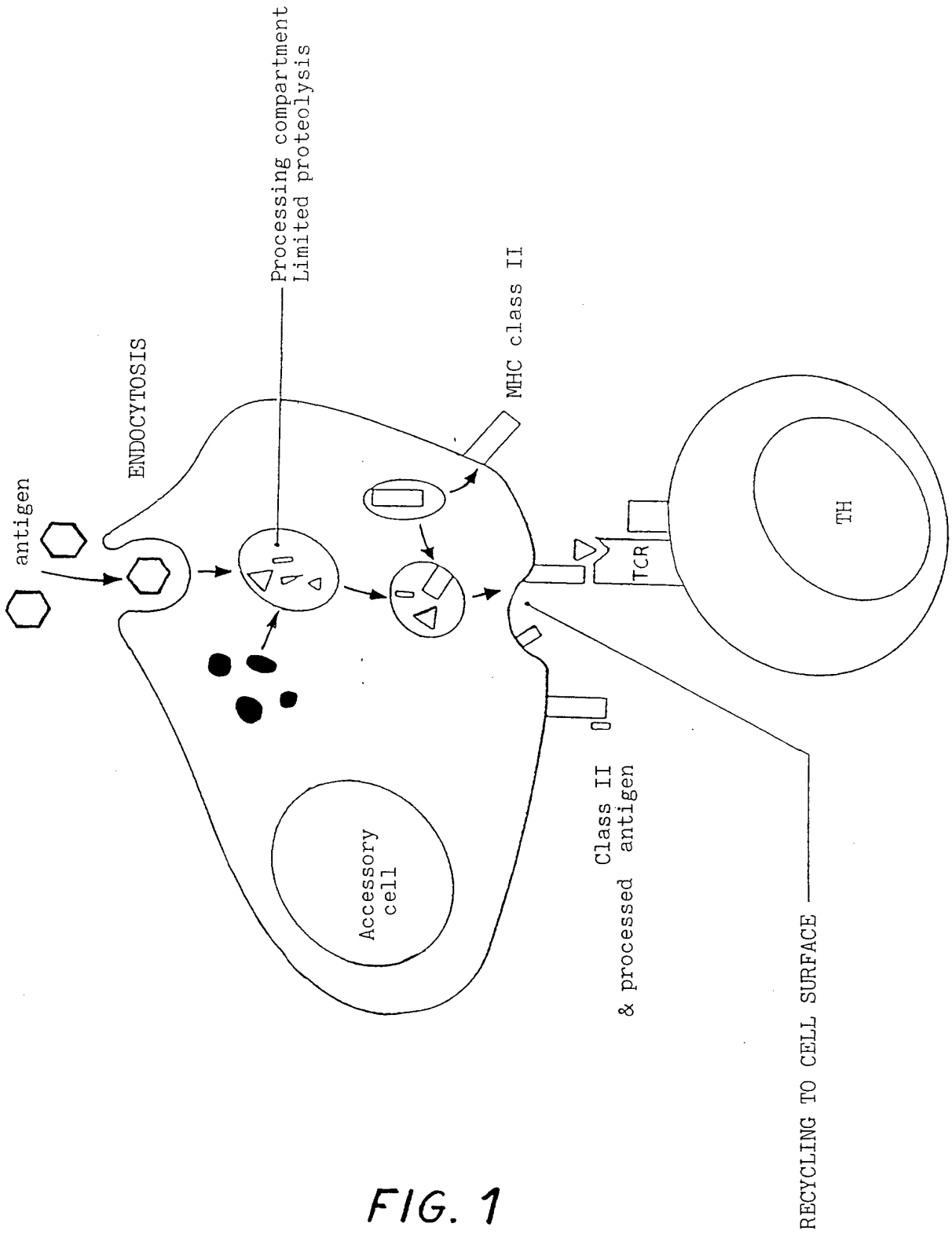

antigen
ENDOCYTOSIS
Processing compartment
Limited proteolysis
MHC class II
TCR
TH
Accessory cell
Class II
& processed antigen
RECYCLING TO CELL SURFACE

FIG. 1

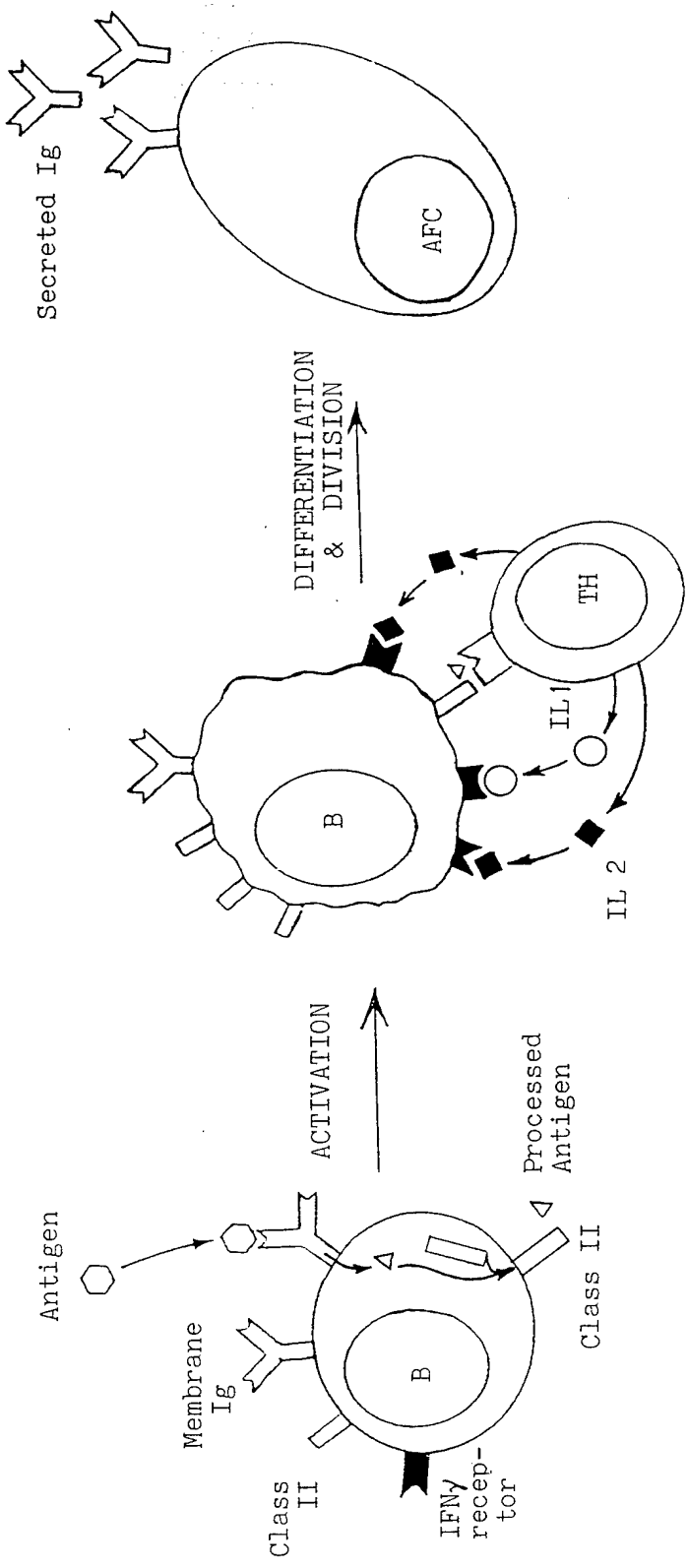

Secreted Ig
AFC
DIFFERENTIATION & DIVISION
TH
IL 1
B
IL 2
ACTIVATION
Antigen
Membrane Ig
B
Processed Antigen
Class II
Class II
IFNγ recep-tor

FIG. 2

IMMUNOGENICITY OF NATIVE AND DENATURED ANTIGEN

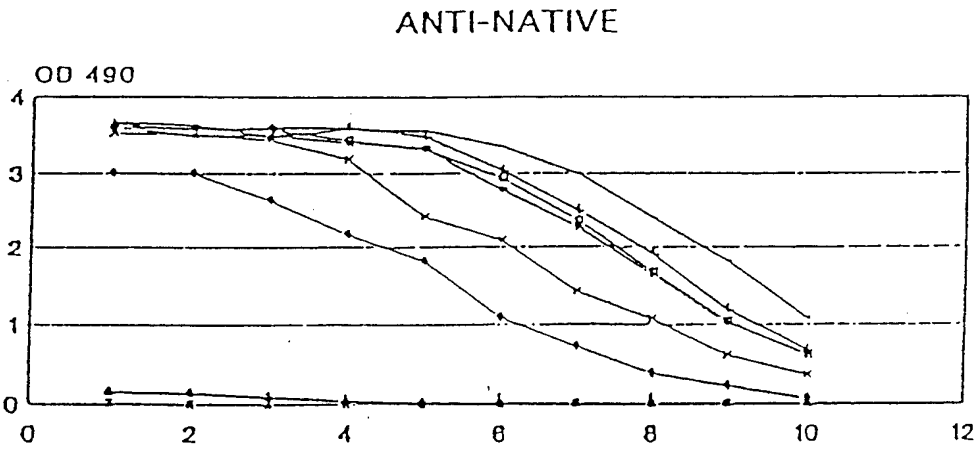


FIG. 3A

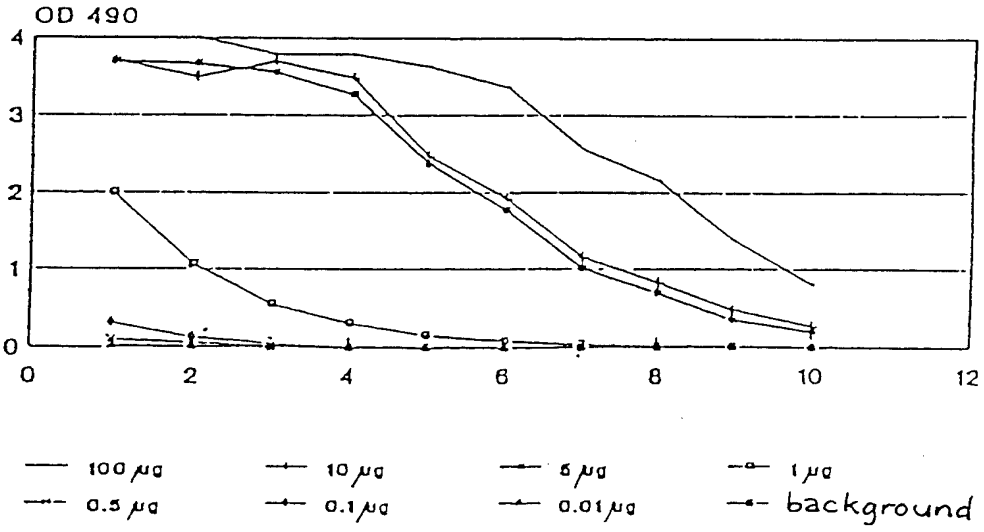


FIG. 3B

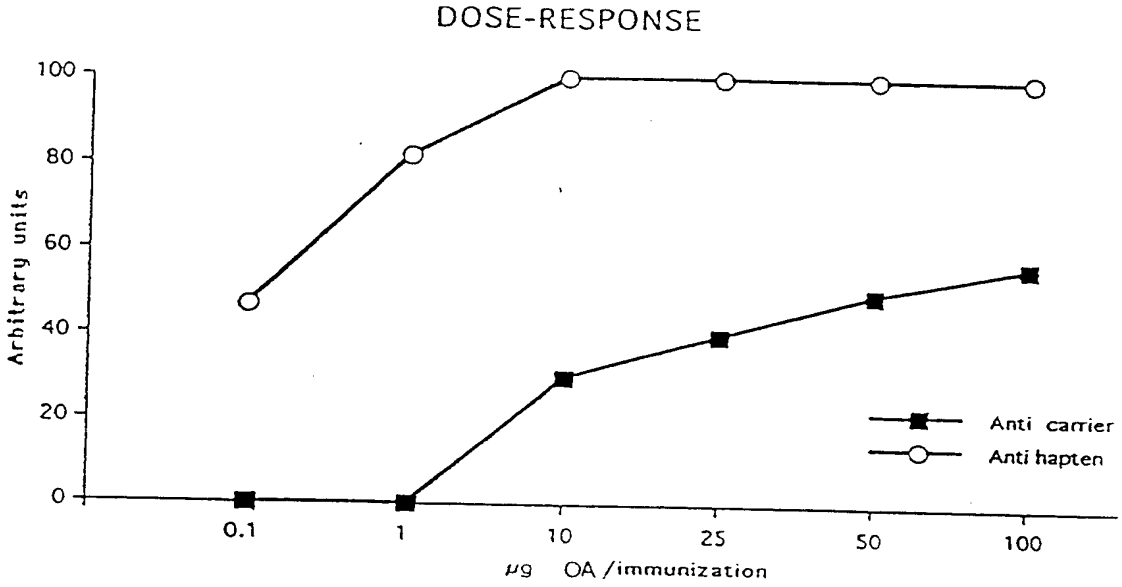

DOSE-RESPONSE
Arbitrary units
100
80
60
40
20
0
0.1
1
10
25
50
100
µg OA /immunization
Anti carrier
Anti hapten

FIG. 4

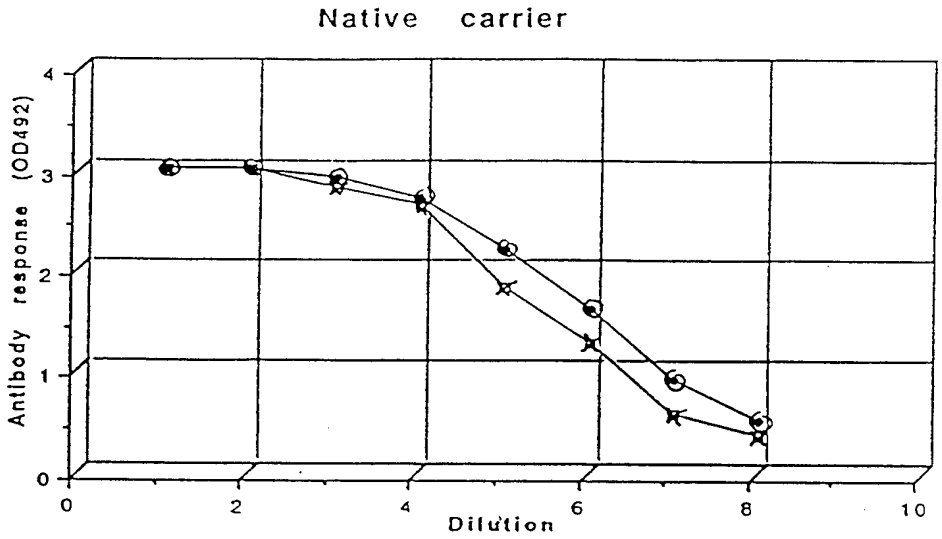

Native carrier
Antibody response (OD492)
0
1
2
3
4
0
2
4
6
8
10
Dilution

FIG. 5A

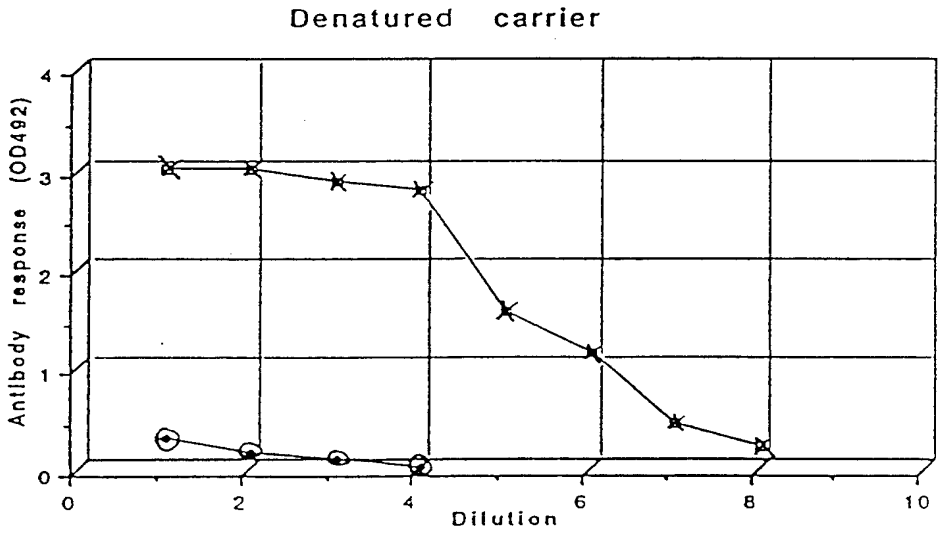

Denatured carrier
Antibody response (OD492)
0
1
2
3
4
0
2
4
6
8
10
Dilution

FIG. 5B

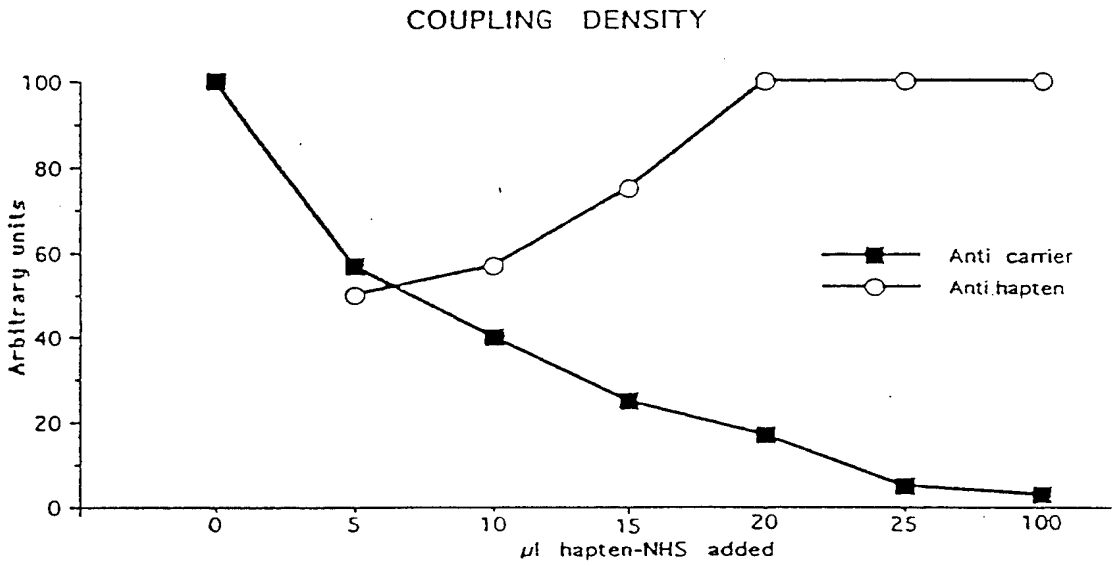

COUPLING DENSITY
Arbitrary units
100
80
60
40
20
0
0
5
10
15
20
25
100
µl hapten-NHS added
Anti carrier
Anti hapten

FIG. 6

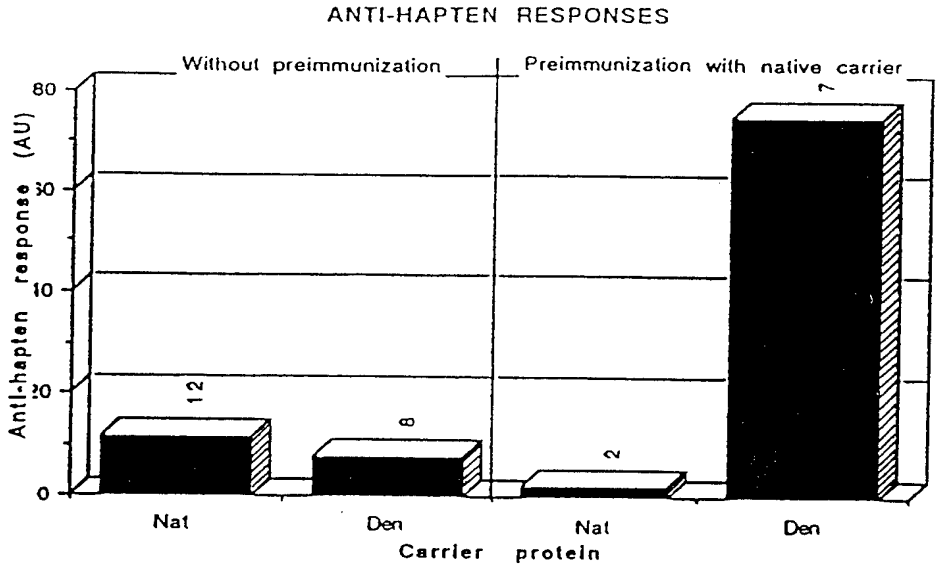

ANTI-HAPTEN RESPONSES
Without preimmunization
Preimmunization with native carrier
Anti-hapten response (AU)
80
30
10
20
0
12
8
2
Nat
Den
Nat
Den
Carrier protein

FIG. 7

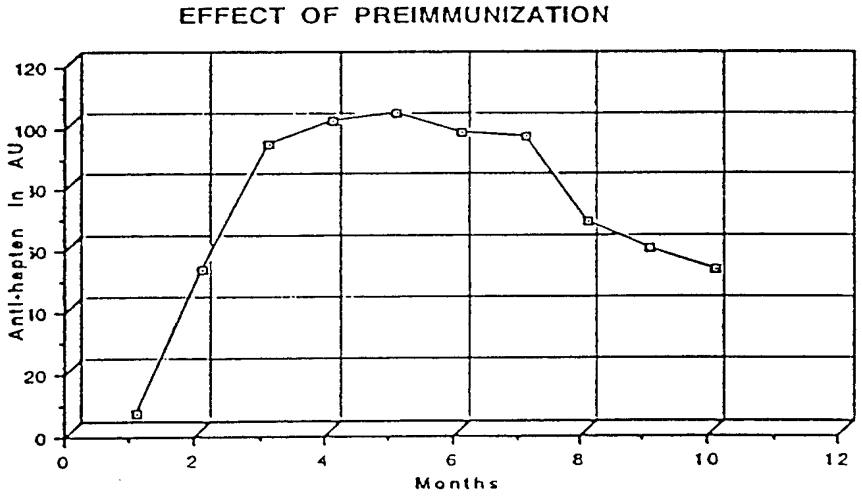

EFFECT OF PREIMMUNIZATION
Anti-hapten In AU
120
100
30
30
10
20
0
0
2
4
6
8
10
12
Months

FIG. 8

INTERNATIONAL SEARCH REPORT

International application No.
PCT/DK 93/00382

A. CLASSIFICATION OF SUBJECT MATTER

IPC5: A61K 39/395, C12P 21/08, C07K 15/28
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC5: A61K, C12P, C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

SE,DK,FI,NO classes as above

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

MEDLINE, WPI

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	EP, A1, 0429816 (F. HOFFMANN-LA ROCHE AG), 5 June 1991 (05.06.91), page 4, line 49 - page 5, line 32; page 5, line 41 - page 6, line 11; page 11, claims	1,3-10
Y	SCIENCE, Volume 249, July 1990, H.M. Etlinger et al, "Use of Prior Vaccinations for the Development of New Vaccines" page 423 - page 425	1,3-10
A	WO, A2, 8906974 (PRAXIS BIOLOGICS, INC.), 10 August 1989 (10.08.89), page 14 - page 16; page 30 - page 32, line 8	1,3-10

[X] Further documents are listed in the continuation of Box C. [X] See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" erlier document but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
1 March 1994	03 -03- 1994
Name and mailing address of the ISA/ Swedish Patent Office Box 5055, S-102 42 STOCKHOLM Facsimile No. +46 8 666 02 86	Authorized officer Carl Olof Gustafsson Telephone No. +46 8 782 25 00

INTERNATIONAL SEARCH REPORT

International application No.
PCT/DK 93/00382

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	EP, A1, 0427347 (ENIRICERCHE S.P.A.), 15 May 1991 (15.05.91)	1-10
	--	
A	EP, A1, 0378881 (ENIRICERCHE S.P.A.), 25 July 1990 (25.07.90)	1,5-8
	--	
Y	METHODS IN ENZYMOLOGY, Volume 70, 1980, B. Erlanger , "The Preparation of Antigenic Hapten-Carrier Conjugates: A Survey" page 85 - page 104	1,3-10
	--	
X	Dialog Information Services, File 351, WPIL, Dialog accession no. 009259776, TOSHIBA KK: "Method of detecting gene by reacting sample with antibody to single stranded oligo-nucleotide - of given base sequence, then detecting presence of antigen-antibody reaction", & JP 4286957 A 921012 9247 (Basic)	1,3,6,9
Y		1,3-10
	--	
X	EP, A2, 0106285 (OTSUKA PHARMACEUTICAL CO., LTD.), 25 April 1984 (25.04.84), see claims and page 14	1
Y		1,3-10
	--	

INTERNATIONAL SEARCH REPORT

Information on patent family members

28/01/94

International application No.
PCT/DK 93/00382

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
EP-A1- 0429816	05/06/91	AU-B-	637841	10/06/93
		AU-A-	6557190	09/05/91
		JP-A-	3173830	29/07/91
WO-A2- 8906974	10/08/89	AU-B-	634153	18/02/93
		AU-A-	3065489	25/08/89
		EP-A-	0399001	28/11/90
EP-A1- 0427347	15/05/91	US-A-	5196512	23/03/93
EP-A1- 0378881	25/07/90	SE-T3-	0378881	
		CA-A-	2006700	17/07/90
		DE-T-	68907045	02/12/93
		JP-A-	2221295	04/09/90
EP-A2- 0106285	25/04/84	CA-A-	1236016	03/05/88
		JP-A-	59225119	18/12/84
		AU-B-	556941	27/11/86
		AU-A-	1995383	12/04/84
		JP-A-	59067224	16/04/84